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Genetic diversity of *Anaplasma* species major surface proteins and implications for anaplasmosis serodiagnosis and vaccine development

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Abstract

The genus *Anaplasma* (Rickettsiales: Anaplasmataceae) includes several pathogens of veterinary and human medical importance. An understanding of the diversity of *Anaplasma* major surface proteins (MSPs), including those MSPs that modulate infection, development of persistent infections, and transmission of pathogens by ticks, is derived in part, by characterization and phylogenetic analyses of geographic strains. Information concerning the genetic diversity of *Anaplasma* spp. MSPs will likely influence the development of serodiagnostic assays and vaccine strategies for the control of anaplasmosis.

Keywords: *Anaplasma*, major surface proteins, diagnosis, vaccination

Introduction

The genus *Anaplasma* (Rickettsiales: Anaplasmataceae) contains obligate intracellular organisms found exclusively within membrane-bound vacuoles in the cytoplasm of both vertebrate and invertebrate host cells (Dumler *et al.*, 2001). The genus *Anaplasma* includes pathogens of ruminants, *A. marginale*, *A. centrale* (*A. marginale* ssp. *centrale*), *A. bovis* (formerly *Ehrlichia bovis*), and *A. ovis*. Also included in this genus is *A. phagocytophilum* (previously recognized as *E. equi*, *E. phagocytophila*, and the human granulocytic ehrlichiosis (HGE) agent), which infects a wide range of hosts including humans, rodents, birds, dogs and cattle, and *A. platys* (formerly *E. platys*) which infects dogs. *Aegyptianella*, which is infective for birds, was originally retained as a *genus incertae sedis* due to lack of sequence information, but was recently confirmed to be closely related to *Anaplasma* spp., based

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on 16 S rRNA and *groEL* gene sequences of *Ae. pullorum* (Rikihisa *et al.*, 2003). However, *Aegyptianella* has yet to be formally renamed as a member of the *Anaplasma* genus.

The type species for the genus *Anaplasma*, *A. marginale*, is an obligate intraerythrocytic pathogen that causes bovine anaplasmosis (reviewed by Kocan *et al.*, 2003). Anaplasmosis is endemic in tropical and sub-tropical regions of the world and the disease causes considerable economic losses globally to both dairy and beef industries worldwide (reviewed by Kocan *et al.*, 2003). Biological transmission of *A. marginale* is effected by ticks and approximately 20 species of ticks have been incriminated as vectors worldwide, while mechanical transmission occurs when infected blood is transferred to susceptible cattle by biting flies or blood-contaminated fomites (reviewed by Kocan *et al.*, 2004). Many geographic strains of *A. marginale* have been identified that differ in biology, morphology, protein sequences, antigenic characteristics, and infectivity for ticks (reviewed by de la Fuente *et al.*, 2001a; Kocan *et al.*, 2003, 2004). Bovine anaplasmosis often results in the development of mild to severe anemia and icterus without hemoglobinemia and hemoglobinuria. Clinical symptoms may include fever, weight loss, abortion, lethargy, icterus, and often death in animals over 2-year old (reviewed by Kocan *et al.*, 2003, 2004). Cattle that survive acute infection develop persistent infections characterized by cyclic low-level rickettsemia (Kieser *et al.*, 1990). Persistently infected or carrier cattle serve as reservoirs of *A. marginale* and provide a source of infective blood for both mechanical transmission and biological transmission by ticks.

Anaplasma centrale is less pathogenic for cattle than *A. marginale*, is only occasionally associated with clinical disease, and is presently used as a live vaccine in Israel, Australia, Africa, and South America (reviewed by Bock *et al.*, 2003). *A. ovis*, a pathogen of wild and domestic sheep, does not establish persistent infection or cause disease in cattle (reviewed by Kocan *et al.*, 2003).

Anaplasma phagocytophilum causes human granulocytic anaplasmosis (HGA), tick-borne fever of ruminants, and equine and canine granulocytic anaplasmosis (Dumler *et al.*, 2001). HGA was first described in the United States in 1994 and subsequently reported in Europe and South and North America. Since this time, *A. phagocytophilum* had become a predominant form of anaplasmosis and among the most common tick-borne pathogens in the United States and Europe (Massung and Slater, 2003; Parola, 2004). Human anaplasmosis is characterized by fever, headache, myalgia, and malaise, as well as leukopenia, thrombocytopenia, and elevated levels of C-reactive protein and liver transaminases, both of which are evidence of hepatic injury (reviewed by Carlyon and Fikrig, 2003). Although the disease is usually self-limiting, severe complications can result, including prolonged fever, shock, seizures, pneumonitis, acute renal failure, hemorrhage, rhabdomyolysis, and opportunistic infections that may result in death (reviewed by Carlyon and Fikrig, 2003).

Anaplasma phagocytophilum is transmitted by tick species belonging to the *Ixodes persulcatus* complex, including *I. scapularis* and *I. pacificus* in the United States, *I. ricinus* in Western Europe, and *I. persulcatus* in eastern Europe and Asia, but other tick species may subsequently prove to be vectors (de la Fuente *et al.*, 2004a). *A. phagocytophilum* infections are maintained in nature, at least in part, in small- and medium-sized mammals including white-footed mice (*Peromyscus leucopus*), raccoons (*Procyon lotor*), and gray squirrels (*Sciurus carolinensis*) (Telford *et al.*, 1996; Levin *et al.*, 2002; Petrovec *et al.*, 2002). Wild rabbits, birds, and cats have been also implicated in the epidemiology of *A. phagocytophilum* (Daniels *et al.*, 2002; Goethert and Telford, 2003; Lappin *et al.*, 2004). Evidence suggests that subclinical persistent infections occur in domestic and wild ruminants, including white-tailed, red, and roe deer (Dumler *et al.*, 2001; de la Fuente *et al.*, 2004b; Polin *et al.*, 2004). The clinical and host diversity of *A. phagocytophilum* suggest the presence of genetic differences among these strains that have not been characterized and may contribute to the diversity of complex infection–transmission networks that impact the epidemiology of the disease.

Recent reports have demonstrated concurrent infections of *Anaplasma* spp. in ruminants and ticks (de la Fuente *et al.*, 2004a; Hofmann-Lehmann *et al.*, 2004; Lin *et al.*, 2004). The establishment of concurrent *Anaplasma* spp. infections in reservoir hosts is likely to increase the risk of pathogen transmission among wildlife, domestic animals, and humans, and emphasizes the need for effective diagnostic assays and vaccines for the control of anaplasmosis.

Several MSPs have been identified in *Anaplasma* spp., which have been most extensively characterized in *A. marginale* (Palmer *et al.*, 1985; reviewed by de la Fuente *et al.*, 2001a; Kocan *et al.*, 2003, 2004). Some of these MSPs have been tested for use in serodiagnostic assays and vaccines. Sequence information that allows assessment of genetic diversity among *Anaplasma* strains have been obtained thus far for MSP1a, MSP2, MSP4, and MSP5 and therefore this review will focus on genetic diversity of *Anaplasma* spp. using these MSPs (Table 1). The implications of the results of these sequence analyses for the diagnosis and control of anaplasmosis will be discussed.

Anaplasma major surface proteins

MSP1a

The gene *msp1α*, encoding for MSP1a, has only been identified thus far in *A. marginale* despite attempts to clone this gene from *A. centrale*, *A. ovis*, and

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Table 1. Geographic and host distribution of *Anaplasma* strains from which MSP sequences were derived for the analysis

Anaplasma species	MSP1a			MSP2			MSP4			MSP5		
	N	Country of origin	Host	*N*	Country of origin	Host	*N*	Country of origin	Host	*N*	Country of origin	Host
A. centrale	0	NA	NA	3	Israel	Bovine	1	Israel	Bovine	1	Israel	Bovine
A. ovis	0	NA	NA	8	USA	Ovine	4	Italy USA	Ovine	0	NA	NA
A. phagocytophilum	0	NA	NA	8	Norway Switzerland UK USA	Bovine Ovine Equine Human	21	Germany Italy Norway Poland Spain Switzerland USA	Bovine Ovine Equine Human Canine White-tailed deer Roe deer Bison Donkey	1	USA	Human
A. marginale	55	Argentina Australia Brazil Canada Italy Israel Mexico Puerto Rico USA	Bovine Bison	7	USA	Bovine	76	Argentina Australia Brazil Canada Italy Israel Kenya Mexico Puerto Rico Spain South Africa Switzerland USA Zimbabwe	Bovine Bison Tick	3	Cuba Brazil USA	Bovine
Total	55	9	2	26	5	4	102	17	10	5	4	2

NA, Not analyzed because sequence information is not available.

A. phagocytophilum (Lew *et al.*, 2002; unpublished results). MSP1a is one of the six MSPs that have been identified in *A. marginale* from bovine erythrocytes and found to be conserved on *A. marginale* derived from ticks and cultured tick cells (reviewed by Kocan *et al.*, 2003, 2004). MSP1a is a part of the MSP1 complex composed of a heterodimer of two structurally unrelated polypeptides: MSP1a, which is encoded by a single gene and MSP1b, which is encoded by at least two genes, *msp1β1* and *msp1β2* (Barbet *et al.*, 1987; Viseshakul *et al.*, 2000; Camacho-Nuez *et al.*, 2000; Bowie *et al.*, 2002). The MSP1a of *A. marginale* geographic strains differs in molecular weight because of a variable number of tandem 28 or 29 amino acid repeats, and has been used for strain identification (Allred *et al.*, 1990; de la Fuente *et al.*, 2001a, 2003a). Within a strain, *msp1α* does not vary and has proved to be a stable genetic marker of the rickettsia through its developmental cycle in cattle and ticks (Bowie *et al.*, 2002).

MSP1a was shown to be an adhesin for bovine erythrocytes and both native and cultured tick cells in microtitre hemagglutination and adhesion assays using recombinant *Escherichia coli* expressing MSP1a (McGarey and Allred, 1994; McGarey *et al.*, 1994; de la Fuente *et al.*, 2001b), and by light and electron microscopy (de la Fuente *et al.*, 2001b). MSP1a was also shown to be involved in the infectivity of *A. marginale* strains for *Dermacentor* spp. (de la Fuente *et al.*, 2001c). Expression of MSP1a was found to be down-regulated in tick cells, as compared with organisms derived from bovine erythrocytes. In addition, both native and recombinant MSP1a were found to be glycosylated, a feature of the repeated N-terminal peptides which appears to contribute to the adhesive properties and thus the function of the protein (Garcia-Garcia *et al.*, 2004a, b, c).

The adhesion domain of MSP1a was identified on the extracellular N-terminal region of MSP1a that contains the repeated peptides (de la Fuente *et al.*, 2003b). We demonstrated by the use of mutant proteins that the repeated peptides of MSP1a were necessary and sufficient to mediate adhesion of recombinant MSP1a to tick cells and bovine erythrocytes, a prerequisite to infection of host cells (de la Fuente *et al.*, 2003b). The adhesive capacity of individual peptides for tick cell extract (TCE) was then evaluated using synthetic peptides representing the predominant repeated sequences (de la Fuente *et al.*, 2003b) and was found to be associated with specific amino acids. Peptides containing acidic amino acids D or E at position 20 bound to TCE, while peptides with a G as the 20th amino acid were not adhesive to TCE. Antibodies produced in rabbits against a synthetic repeated peptide significantly reduced *A. marginale* infection of cultured tick cells, and the neutralization observed was similar to that obtained using antibodies produced against the whole MSP1a recombinant protein (de la Fuente *et al.*, 2003b). Analyses of the tandemly repeated MSP1a peptides of several geographic strains of *A. marginale* revealed a complex relationship between the *msp1α* genotype and the tick-transmissible phenotype of the strain and suggested that both the sequence and conformation of this portion of MSP1a influenced the adhesive properties of the protein (de la Fuente *et al.*, 2003b). The results of these studies reinforced the functional importance of the MSP1a tandem repeats in adhesion, invasion, and transmission of *A. marginale*.

A. marginale MSPs are involved in interactions with both vertebrate and invertebrate hosts (reviewed by de la Fuente *et al.*, 2001a; Kocan *et al.*, 2003, 2004), and therefore are likely to evolve more rapidly than other nuclear genes because they are subjected to selective pressures exerted by host immune systems. While both *msp1α* and *msp4* were found to be stable genetic markers during the multiplication of a given *A. marginale* strain (de la Fuente *et al.*, 2001d; Bowie *et al.*, 2002), they proved to be useful in phylogenetic analysis among geographic *A. marginale* strains.

Initially, we chose two *A. marginale* MSPs, MSP1a and MSP4, for phylogenetic analysis and these studies provided evidence that *msp1α* is under positive selection pressure (de la Fuente *et al.*, 2003a). Phylogenetic analysis of *A. marginale* was first performed on strains from the USA, using *msp1α* and *msp4* genes and derived protein sequences (de la Fuente *et al.*, 2001d). Results of these analyses strongly supported a southeastern clade of *A. marginale* comprising Virginia and Florida isolates. Furthermore, analysis of 16 S rDNA fragment sequences from the tick vector of *A. marginale*, *D. variabilis*, from various areas of the USA was performed, which suggested co-evolution of the vector and pathogen (de la Fuente *et al.*, 2001d).

Subsequent phylogenetic studies of MSP1a DNA and protein sequences from 20 New World strains of *A. marginale* failed to provide phylogeographic resolution (de la Fuente *et al.*, 2002). Most of the variations in MSP1a sequences appeared unique for a given strain. However, similar DNA sequence variation in MSP1a was detected within strains from Idaho and Florida and from Idaho and Argentina. These results suggested that MSP1a sequences may be rapidly evolving and questioned the use of MSP1a sequences for defining geographic strains of *A. marginale*. These findings were then confirmed in phylogenetic analyses of MSP1a DNA and protein sequences of 13 strains from Oklahoma, in comparison with seven Latin American and 13 strains from the USA, which demonstrated no geographic clustering (de la Fuente *et al.*, 2003a). From these studies, we concluded that MSP1a is not useful as a marker for the characterization of geographic strains of *A. marginale*. The genetic heterogeneity observed among strains of *A. marginale* within Oklahoma, and within other endemic regions like Oregon (Palmer *et al.*, 2001), Minas Gerais in Brazil (de la Fuente *et al.*, 2004c), Castilla-La Mancha in Spain (de la Fuente *et al.*, 2004b), Kansas (Palmer *et al.*, 2004) and

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(A)

Repeat form	Encoded sequence
A	DDSSSASGQQQESSVSSQSE-ASTSSQLG-
B	A*****G**********DQ*********
C	A*****G**********GQ*********
D	A**************************G
E	A***************************
F	T******************GQ*********
G	******************GQ******S**
H	T*****************GQ******S**
I	******************GQ*********
J	A***L*G**********DQ*********
K	A*G***G**********DQ*********
L	AG****D**********DQ*********
M	A*****************GQ*********
m	A*****************GQ******S**
N	T*****************DQ*********
O	----**G**********DQ*********
P	T************G****GQ**H*A*S**
Q	A*****************DQ*********
R	A*****G***H********DQ*******W*
S	A*G***G**********DQ*********
T	AG****G**********DQ*********
U	******************DQ*********
V	A*****G***-********DQ*********
W	T******************GQ******SR*
α	A*******------*L***GQ*********
ß	T*****GD***G*G*****GQ*********
Γ	T*****************D**********
π	A*****G**********GQ******F**
Σ	A*****G**********************
σ	A*****G******I*****DH*********
μ	A*******L*********GQ*********
τ	T*************L*P*GQ*********
Φ	T****************************
1	SG***********L**GGQ*********
2	T***************P**GQ*********
3	A**************L***GQ*********
4	T**************L***GQ*********
5	A*****************D**********
6	A****************H***********
7	T****************H***********
8	A*G***GD**********G******S**

Fig. 1. Continued.

Sicily in Italy (de la Fuente *et al.*, 2005b) could be explained by cattle movement and maintenance of different genotypes by independent transmission events, due to infection exclusion of *A. marginale* in cattle and ticks which results in the establishment of only one genotype per animal (de la Fuente *et al.*, 2003a). However, recent results have documented the low frequency appearance of animals infected with two *A. marginale* strains in a cattle herd with high prevalence of infection (Palmer *et al.*, 2004). The *A. marginale msp1*α genotypes in animals infected with two strains were not closely related and may reflect a situation similar to the *A. centrale*/*A. marginale* co-infection reported in vaccinated cattle (Shkap *et al.*, 2002b). When cattle movement imports a new *A. marginale* genotype, it becomes established and maintained by mechanical and/or biological transmission to susceptible cattle. Transmission of *A. marginale* strains appears to be stochastic, at least within a herd with a high prevalence of infection (Palmer *et al.*, 2004). These results predict that genotypic variation of *A. marginale* strains would be minimal in regions with few cattle/*A. marginale* introductions, while a highly heterogeneous population of *A. marginale* genotypes would be expected to occur in regions with extensive cattle movement, such as Oklahoma.

Phylogenetic analysis of 55 MSP1a sequences derived from bovine and bison in nine countries in North and South America, Asia, Australia, and Europe (Table 1) corroborated these predictions. A high degree of variation was found among MSP1a sequences (Fig. 1 and Table 2). Although MSP1a sequences failed to provide phylogeographic information, the sequences of Australian strains clustered together (bootstrap support (bs)=94%; Fig. 2), supporting a genetically more homogeneous *A. marginale* population in this region with fewer cattle introductions (Lew *et al.*, 2002). However, phylogeographic

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(B)

A. marginale isolate	Structure of MSP1a tandem repeats	No. of repeats
Florida	A B B B B B B B	8
Idaho	D D D D D E	6
Virginia	A B	2
Washington	B B B C	4
Wetumka, OK	K C H	3
Cushing, OK	L C B C	4
Cushing 2,OK	S N N F H	5
Glencoe 1, OK	S F N F H	5
Glencoe 2, OK	B M F H	4
Glencoe 3, OK	T B C	3
Stillwater, OK	S F F F H	5
Stillwater 2, OK	L B C C	4
Stillwater 68, OK	S B M F H	5
Oklahoma City, OK	U	1
Okmulgee, OK	S B V C	4
Stigler, OK	T B B C	4
Pawhuska, OK	I H	2
New Castle, OK	L B C B	4
St. Maries, ID	J B B	3
California	B B C	3
Okeechobee, FL	L B C B C	5
Mississippi	D D D D E	5
Missouri	B B B B	4
Illinois	M N B M H	5
Texas	O B M P	4
Texas 198	B B m B m	5
South Dakota	A F H	3
Oregon	A F H	3
Kansas 3261	B B	2
Kansas 4102	B B B	3
Kansas 2267	B B B B	4
Kansas 0141	B B B B B	5
Kansas 0063	B B B B B B	6
Kansas 5076	D D D D D	5
Kansas 7042	D D E	3
Kansas 4318	D D D D D E	6
Kansas 2070	D D D D D D E	7
Kansas 7030	D D D D D D D D D E	10
Kansas 0050	E M	3
Canadian bison	D Q Q R	4
U.S. bison (buffalo)	K B M F W	5
Yucatán	T C B B C B π	7
Mexico	α ß ß Γ	4
Morelos	α ß ß Γ	4
Veracruz	α ß ß Γ	4
Brazil 9	α ß τ M	4
Brazil 12	α ß ß N	4
Brazil 5	C F N	3
Brazil	B B Q σ μ	5
Virasoro	Σ B Q B C	5
Salta	B B M	3
Puerto Rico	E Φ Φ Φ Φ Φ	6
Israel tailed 1FM3	1 F M 3	4
Israel tailed 12M3	1 2 M 3	4
Israel non-tailed	1 4	2
Italy 30	M M M Q	4
Italy 31	M M M Q	4
Italy 32	5 Γ Γ Γ	4
Italy 16	5 Φ Φ Φ	4
Italy 6	Q M Q Q M	5
Italy 8	Q N N N	4
Italy 47	6 7 7 7	4
Australia Darwin	8	1
Australia WA	8	1
Australia F12	8	1
Australia F72	8	1

Fig. 1. Sequence of MSP1a tandem repeats in strains of *A. marginale*. (A) The one letter amino acid code was used to depict the different sequences found in MSP1a repeats. Asterisks indicate identical amino acids. Gaps indicate deletions/insertions. (B) The structure of the MSP1a repeat regions was represented for strains of *A. marginale* from North America (Allred *et al.*, 1990; Palmer *et al.*, 2001, 2004; de la Fuente *et al.*, 2003a, d), Latin America (de la Fuente *et al.*, 2002, 2004c), Italy (de la Fuente *et al.*, 2005b), Australia (Lew *et al.*, 2002), and Israel (Shkap *et al.*, 2002b) using the repeat forms described in (A).

Table 2. Protein similarity range between *Anaplasma* strain MSPs

Pairwise comparison	Similarity range (%)			
	MSP1a	**MSP2**	**MSP4**	**MSP5**
A.c–A.c	ND	92.7–95.2	ND	ND
A.o–A.o	ND	93.6–99.8	99.6–100	ND
A.p–A.p	ND	89.0–99.7	91.5–100	ND
A.m–A.m	41.5–99.0	83.3–96.3	97.9–100	97.1–99.0
A.c–A.o	ND	79.3–81.4	89.0–89.4	ND
A.c–A.p	ND	28.9–33.1	58.5–59.2	66.8
A.c–A.m	ND	74.9–83.3	88.3–89.0	91.4–92.9
A.o–A.p	ND	27.7–29.0	58.2–59.6	ND
A.o–A.m	ND	82.5–89.5	95.7–96.8	ND
A.p–A.m	ND	25.5–28.6	56.9–59.2	64.9–65.4

Abbreviations: A.c, *A. centrale*; A.o, *A. ovis*; A.p, *A. phagocytophilum*; A.m, *A. marginale*, ND, not determined because sequence information was not available.

resolution was not obtained in all the other regions of the world analyzed, suggesting multiple introductions of *A. marginale* strains from different geographic locations.

MSP2

MSP2 is an immunodominant outer membrane protein with orthologs in all *Anaplasma* spp. studied thus far (Palmer *et al.*, 1998; Shkap *et al.*, 2002a; Lin *et al.*, 2004). In *A. marginale*, *A. centrale*, and *A. ovis*, MSP2 is encoded by a multigene family (Palmer *et al.*, 1998; Shkap *et al.*, 2002a), while the corresponding gene in *A. phagocytophilum* is encoded by a single gene, different from the antigenically-related *p44* multigene family (Lin *et al.*, 2004). Cattle and ticks become persistently infected with *A. marginale* and antigenic variation of MSP2 occurs during these persistent infections (French *et al.*, 1998, 1999; de la Fuente and Kocan, 2001). Multiple antigenic variants arise as the result of combinatorial gene conversion into a single expression site (Brayton *et al.*, 2002). This mechanism of antigenic variation has been suggested to allow *A. marginale* to evade the bovine immune response, thus contributing to the maintenance of persistent infections in cattle (French *et al.*, 1998, 1999). Male ticks also become persistently infected with *A. marginale* (Kocan *et al.*, 1992a, b). Male ticks thus also serve as a reservoir host and they are capable of transmitting the pathogen to multiple hosts (reviewed by Kocan *et al.*, 2003, 2004). MSP2 variants appear on *A. marginale* during tick feeding on multiple hosts suggesting that antigenic variation of MSP2 in *D. variabilis* is independent of the bovine immune response (de la Fuente and Kocan, 2001).

Phylogenetic analysis of MSP2 sequences provided some phylogenetic but not phylogeographic information (Lin *et al.*, 2004; de la Fuente *et al.*, 2005a; Fig. 3). The analysis of 26 MSP2 sequences of *A. marginale*, *A. centrale*, *A. ovis*, and *A. phagocytophilum* strains, derived from infected mammalian human and non-human hosts in different countries (Table 1), differentiated between *Anaplasma* species and between the *A. phagocytophilum* strains, derived from ruminant and non-ruminant hosts (Fig. 3). These findings suggest that the function(s) of MSP2 may vary for *A. phagocytophilum* and *A. marginale*/*A. centrale*/*A. ovis* and support the hypothesis that *A. phagocytophilum* strains from ruminants could share some common characteristics, including reservoir hosts and pathogenicity, which may differ from strains that infect humans (de la Fuente *et al.*, 2005a).

MSP4

MSP4, also an immunodominant outer membrane protein with orthologs in all *Anaplasma* spp. examined thus far (de la Fuente *et al.*, 2002, 2005a, b; Molad *et al.*, 2004), is a highly conserved protein encoded by a single gene (Table 2). Although the function of this protein is unknown for any *Anaplasma* spp., it may be conserved among species. Analysis of codon and amino acid changes over the MSP4 phylogeny evidenced that *msp4* is not under positive selection pressure (de la Fuente *et al.*, 2003a). Phylogenetic analyses using MSP4 sequences have provided phylogenetic and phylogeographic information for New World strains of *A. marginale* with two outgroup taxa (*A. centrale* and *A. ovis*) (de la Fuente *et al.*, 2001d, 2002, 2003a, 2004c). When phylogenetic analyses were conducted with 102 MSP4 sequences of *A. marginale*, *A. centrale*, *A. ovis*, and *A. phagocytophilum* strains derived from infected mammalian hosts and ticks in different countries (Table 1), phylogeographic information was not obtained for *A. marginale* strains, but the analysis did differentiate between *Anaplasma* species (Fig. 4). These results support those obtained with MSP1a and indicate that MSP4 is not a good genetic marker for global phylogeographic analysis of *A. marginale* strains, but may still be useful for strain comparison in some regions.

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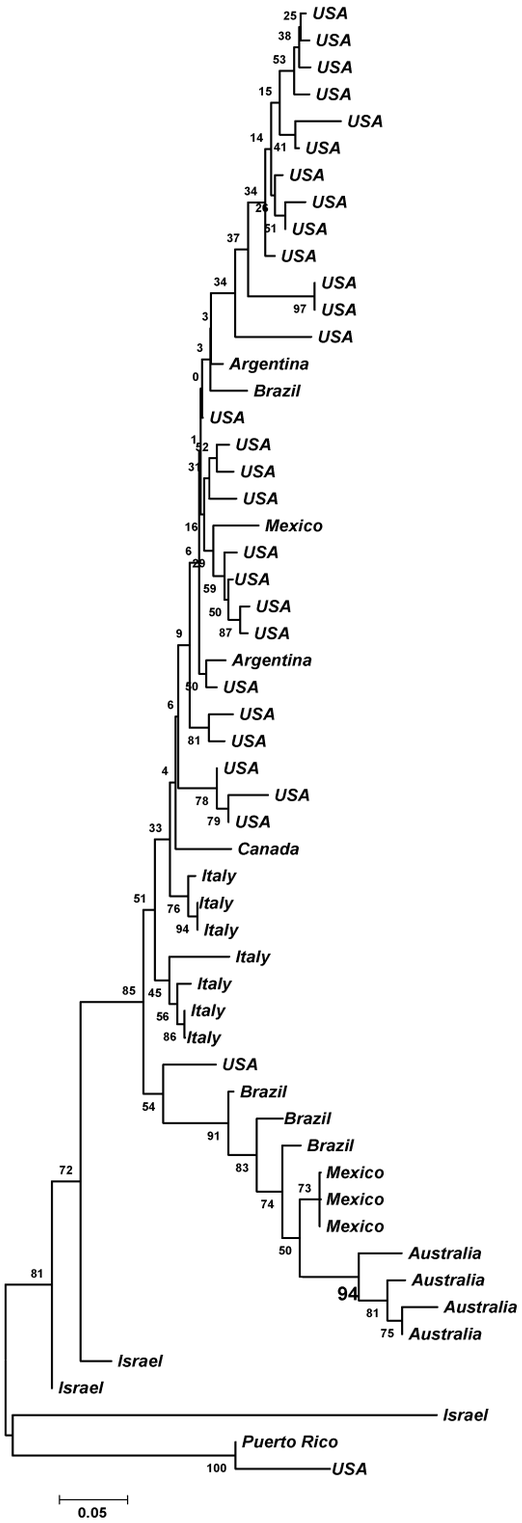

Fig. 2. Phylogenetic analysis using Mega 2 of *A. marginale* strains based on the MSP1a protein sequences using the NJ algorithm with Poisson correction and bootstrap analysis of 1000 replicates. Numbers on the branches indicate percent support for each clade. Strains were coded according to the country of origin to emphasize the geographic distribution of the strains. Sequence information was derived from the references in Fig. 1 legend.

MSP5

MSP5 is an immunodominant protein encoded by a single gene, which has been identified in *A. marginale*, *A. centrale*, *A. ovis*, and *A. phagocytophilum* (Visser *et al.*, 1992; Molad *et al.*, 2004; Dreher *et al.*, submitted for publication). It is a highly conserved protein (Table 2), which, as in the case of MSP4, may have a conserved but currently unknown function. A preliminary phylogenetic analysis of five MSP5 sequences (Table 1) resulted in the differentiation of *Anaplasma* spp. but did not allow for the assessment of its use for phylogeographic analysis of *Anaplasma* strains (Fig. 5).

Anaplasma MSPs and the immunodiagnosis of anaplasmosis

A competitive ELISA (cELISA) has been used for the diagnosis of *Anaplasma* infection in various ruminants including cattle, ovine, and deer (Ndung'u *et al.*, 1995; Knowles *et al.*, 1996; de la Fuente *et al.*, 2004b). The cELISA currently used for the diagnosis of bovine anaplasmosis, developed by Knowles *et al.* (1996), is based on the use of the monoclonal antibody (Mab) ANAF16C1 that recognizes MSP5 in *A. marginale*, *A. centrale*, and *A. ovis* (Visser *et al.*, 1992). However, recent findings suggest that this MSP5 ELISA, currently commercialized by VMRD, Inc. (Pullman, WA, USA), may also recognize *A. phagocytophilum* antibodies in infected cattle (Dreher *et al.*, submitted for publication). The MSP5 sequence is highly conserved and thus similar among strains of *A. marginale*, as well as between *A. marginale*, *A. centrale*, and *A. phagocytophilum* (Table 2). The cross-reactivity of the MSP5 test with multiple species of *Anaplasma* has been confirmed through the identification of common regions defined to be essential for ANAF16C1 reactivity (Munodzana *et al.*, 1998). Thus, the MSP5 cELISA does not differentiate *Anaplasma* species in regions where co-infection with *A. phagocytophylum* and *A. marginale* or *A. centrale* occurs (de la Fuente *et al.*, 2004a; Hofmann-Lehmann *et al.*, 2004; Lin *et al.*, 2004). Although a second test specific for *A. phagocytophilum* (for example the immunofluorescence HGE IFA Antibody Test Kit; Fuller Laboratories, Fullerton, CA, USA) or *A. centrale* (Molloy *et al.*, 2001) may be used, the possibility of cross-infection cannot be ruled out when using these assays in these regions. An ELISA based on recombinant MSP5 for indirect *A. marginale* antibody detection was developed by Morzaria *et al.* (1999) and is commercialized by Svanova Biotech AB (Uppsala, Sweden) but the assay has not been evaluated for cross-reactivity with other *Anaplasma* species. However, it should be possible to explore the possibility of developing serological tests for the differential detection of *Anaplasma* infections based on the non-conserved MSP5 regions.

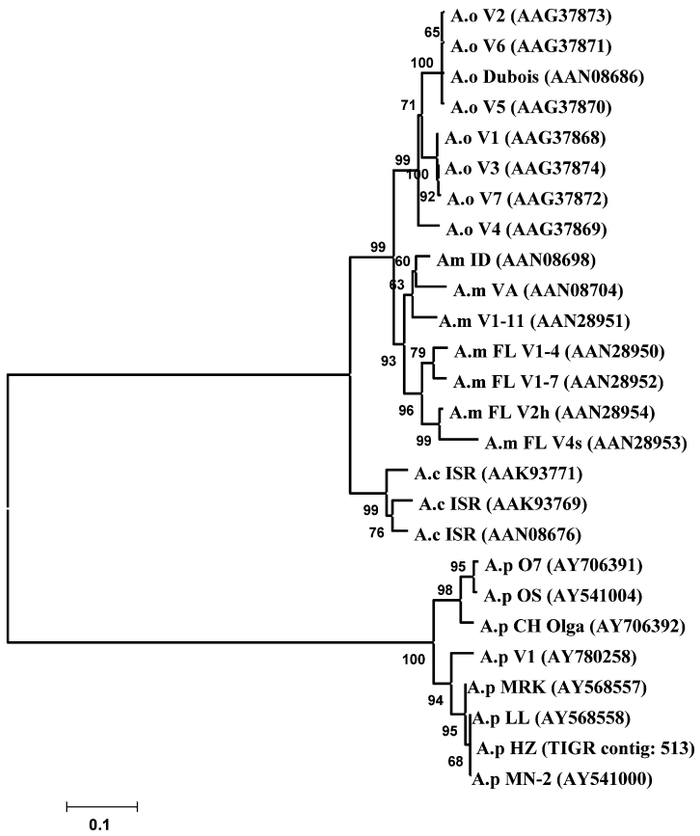

Fig. 3. Phylogenetic analysis using Mega 2 of *Anaplasma* species based on the MSP2 protein sequences using the NJ algorithm with Poisson correction and bootstrap analysis of 1000 replicates. Numbers on the branches indicate percent support for each clade. Sequence GenBank accession numbers are shown in parenthesis. Abbreviations: A.c, *A. centrale*; A.o, *A. ovis*; A.p, *A. phagocytophilum*; A.m, *A. marginale*; V, variant; ID, Idaho, USA; VA, Virginia, USA; FL, Florida, USA; ISR, Israel; CH, Switzerland. The *A. phagocytophilum* HZ genome sequence database is located at www.tigr.org.

Although DNA-based diagnostic methods could be used to identify the pathogen species of *Anaplasma* infections, a serologic test based on MSPs would be more practical for the diagnosis of large number of animals. MSP4, which is also highly conserved among *Anaplasma* spp. (Table 2), would probably give cross-reactive results similar to those obtained with MSP5. According to sequence similarity, the conserved regions of MSP2 may prove useful to discriminate between *A. phagocytophilum* and the other *Anaplasma* spp. (Table 2), but panels of Mabs would have to be generated and characterized to develop a serological test based on MSP2. Recombinant antigens, such as the *A. phagocytophilum* MSP2-related p44, may be used to detect antibodies in infected hosts (Magnarelli *et al*., 2004). MSP1a, although highly variable in the extracellular region between geographic strains of *A. marginale*, could be used to identify infections caused by *A. marginale* because it may prove to be specific for this species. A neutralization epitope, which is conserved among strains of *A. marginale* (Garcia-Garcia *et al*., 2004b), was identified in the repeat regions of MSP1a (Palmer *et al*., 1987). Our group has recently demonstrated that Mabs generated against conserved epitopes in the variable extracellular region of MSP1a were able to recognize geographically distinct strains of *A. marginale* (unpublished results).

Anaplasma MSPs and the development of vaccines for the control of anaplasmosis

The pioneering work of Tebele *et al*. (1991) demonstrated that immunization with *A. marginale* outer membranes induced immunity against clinical disease which correlated with antibody titer to MSPs. Since then, extensive research has been devoted to the identification and testing of *A. marginale* MSPs for their ability to induce a protective immune response in cattle (reviewed by Kocan *et al*., 2003).

Recent research, as reviewed by Palmer (1989) and Palmer *et al*. (1999), has provided much information on the nature of the immune response of cattle to *A. marginale* infection, as well as the identification of key *A. marginale* antigens that appear to play a role in the

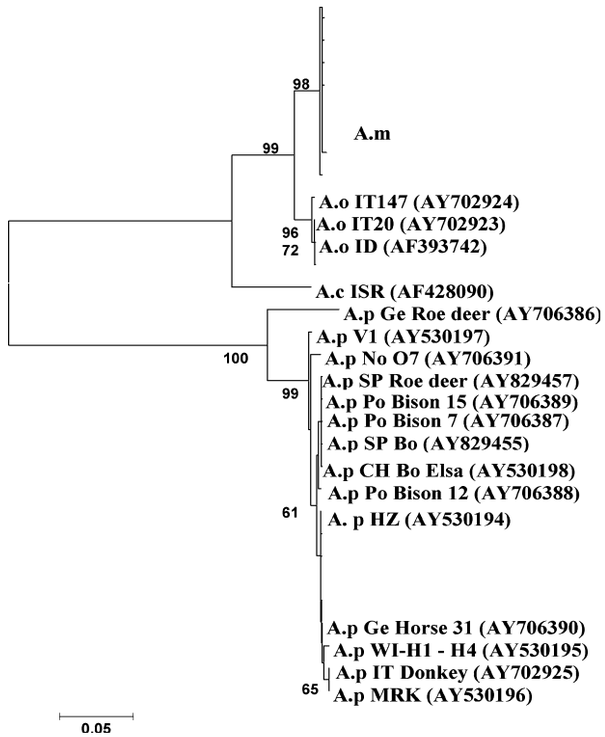

Fig. 4. Phylogenetic analysis using Mega 2 of Anaplasma species based on the MSP4 protein sequences using the NJ algorithm with Poisson correction and bootstrap analysis of 1000 replicates. Numbers on the branches indicate percent support for each clade (>60%). Sequence GenBank accession numbers are shown in parenthesis for *A. phagocytophilum*, *A. ovis*, and *A. centrale*. Strains of *A. marginale* were not indicated to simplify the tree. Sequences for *A. marginale* MSP4 were derived from de la Fuente *et al.* (2001a, d, 2002, 2003a, d, 2004b, c, 2005a, b) or from unpublished results (Israeli strains, AY786993, AY786994, AY787172; Italian strains from Apulia and Calabria regions, AY829458–AY829460; Swiss strains, AY851150; Australian strains, AY665997–AY666003; South African strain, AY666005; Kenyan strain, AY666004; Zimbabwean strains, AY666006–AY666011). Abbreviations: A.c, *A. centrale*; A.o, *A. ovis*; A.p, *A. phagocytophilum*; A.m, *A. marginale*; IT, Italy; ID, Idaho; WI, Wisconsin; ISR, Israel; Ge, Germany; Po, Poland; No, Norway; CH, Switzerland; SP, Spain; V, variant; O, ovine; Bo, bovine; H, human.

immune response. A model for vaccine-induced immunity against *A. marginale* was proposed in which pathogen clearance is affected by antibodies against surface epitopes in combination with macrophage activation for enhanced phagocytosis and killing. The centerpiece of this model is the CD4+ T lymphocyte expressing INF-γ which enhances synthesis of the predominant opsonizing bovine IgG subclass, IgG2, and concomitantly activates macrophages to increase receptor expression, phagocytosis, phagolysomosal fusion, and release of rickettsiacidal nitric oxide. Brown *et al.* (1998a) demonstrated that induction of these responses using purified outer membrane proteins prevented *A. marginale* rickettsemia upon challenge-exposure. CD4+ T lymphocyte clones from protectively immunized cattle were found to be diverse and several clones responded to MSP1a and MSP2 (Brown *et al.*, 1998b, 2001a, b, 2002, 2003, 2004). Furthermore, recent results have indicated that WC1(+) gamma delta T cells recognize *A. marginale* MSP2 through the T cell receptor and may contribute to the immunodominant response to this protein (Lahmers *et al.*, 2005). However, in a recent study, thymectomized calves were able to control acute anaplasmosis after their CD4+ T lymphocytes were selectively depleted by treatment with an anti-CD4 Mab (Valdez *et al.*, 2002). Therefore, although CD4+ T lymphocytes may play a role in controlling *A. marginale* infection, the antibody response appears to be essential.

Limited vaccine trials have been conducted using recombinant MSPs (as reviewed by Kocan *et al.*, 2003), recombinant vaccinia virus expressing *A. marginale* antigens (McGuire *et al.*, 1994) or with naked DNA (Arulkanthan *et al.*, 1999). Thus far, only partial protection has been obtained with recombinant antigens used for vaccination, suggesting that a vaccine containing multiple antigens will probably be required to induce a protective immune response. From vaccination trials and *in vitro* studies, MSP1a and MSP2 have provided the most encouraging results. The *A. marginale* MSP2, although capable of inducing a strong T cell response (Brown *et al.*, 1998b, 2001a, 2003, 2004; Abbott *et al.*, 2004), is antigenically variable in persistently infected cattle, which may reduce the effectiveness of this MSP for use in a

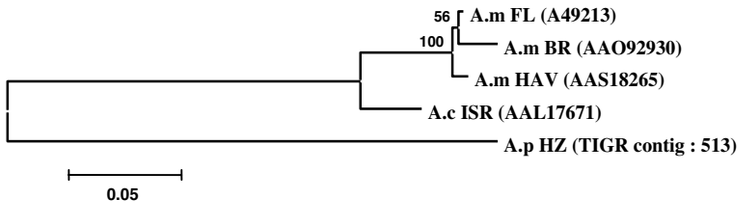

Fig. 5. Phylogenetic analysis using Mega 2 of Anaplasma species based on the MSP5 protein sequence using the NJ algorithm with Poisson correction and bootstrap analysis of 1000 replicates. Numbers on the branches indicate percent support for each clade. Sequence GenBank accession numbers are shown in parenthesis. Abbreviations: A.c, *A. centrale*; A.p, *A. phagocytophilum*; A.m, *A. marginale*, FL, Florida, USA; BR, Brazil; ISR, Israel, HAV, Havana, Cuba. The *A. phagocytophilum* HZ genome sequence database is located at www.tigr.org.

vaccine formulation. MSP2 antigenically variable B-cell epitopes are concentrated in the hypervariable region and are recognized by the immune system of *A. marginale*-infected cattle (Abbott *et al.*, 2004), which results in the selection of new variants that may allow the pathogen to establish persistent infections (French *et al.*, 1998, 1999). Therefore, the effect of the antibody responses against MSP2 most likely would not be neutralizing. Nevertheless, engineered synthetic polypeptides containing MSP2 conserved regions could be evaluated as candidate immunogens.

MSP1a, on the other hand, although variable in the number of repeated peptides, induces strong T cell responses (Brown *et al.*, 2001b, 2002) and contains conserved B cell epitopes in the repeated peptides that are recognized by immunized and protected cattle (Garcia-Garcia *et al.*, 2004b). These results, together with the biological significance of MSP1a function in infection and transmission of *A. marginale*, suggest that MSP1a may be a good candidate for inclusion in vaccines for the control of bovine anaplasmosis. Experiments conducted to evaluate the protection capacity of recombinant MSP1a alone or in combination with whole *A. marginale* antigens from infected cultured tick IDE8 cells demonstrated that a preferential antibody response to MSP1a correlates with lower percent reductions in packed cell volume (PCV) and thus reduced clinical disease (Garcia-Garcia *et al.*, 2004b). Although cattle infected with *A. marginale* mount an immune response against MSP1a, it is likely directed to the MSP1 complex in which MSP1a is covalently linked to MSP1b, perhaps masking some of the MSP1a protective epitopes otherwise exposed in the recombinant antigen. This hypothesis is further supported by the finding that immunization of cattle with *A. marginale* derived from infected erythrocytes in which MSP1a is upregulated with respect to MSP1b and probably exists uncoupled to MSP1b, provided a level of protection comparable to that observed in cattle immunized with recombinant MSP1a and *A. marginale* derived from infected tick cells, in which MSP1a is downregulated and most likely exists only in the MSP1 complex with MSP1b (Garcia-Garcia *et al.*, 2004b). Another factor to be considered for the induction of a protective immune response against *A. marginale* is the role of sugar moieties of MSP1a in the protection properties, which may also contribute to the generation of a neutralizing response in cattle (Garcia-Garcia *et al.*, 2004a).

A. centrale, isolated by Sir Arnold Theiler in the early 1900s, is the most widely used live vaccine strain for control of bovine anaplasmosis (Theiler, 1911). This *A. centrale* strain continues to be used for vaccine production in several areas of the world including Africa, Australia, Israel, and South America (reviewed by Bock *et al.*, 2003; Kocan *et al.*, 2003). As discussed above, MSP1a has not been identified in *A. centrale* despite multiple attempts for its cloning. However, although a protein functionally similar to MSP1a may still exist in *A. centrale*, this result suggests that differences may exist in the mechanism of protection elicited by *A. centrale* and *A. marginale*. Nevertheless, both *A. centrale* and *A. marginale* share immunodominant epitopes that may play a role in the protection induced by *A. centrale* (Shkap *et al.*, 1991, 2002a, b). Bock *et al.* (2003) described the use of a mild strain of *A. marginale* as an alternative to the *A. centrale* vaccine but its efficacy has not been demonstrated yet.

Recently, *A. marginale* proteins with molecular weights ranging from 17 to 43 kDa have been identified for their capacity to induce a protective response in cattle through the induction of IgG2 responses (Riding *et al.*, 2003; Barigye *et al.*, 2004; Hope *et al.*, 2004). These antigens, predicted to be integral membrane proteins, may prove to be new candidate MSPs for vaccination against bovine anaplasmosis.

Recombinant MSPs from other *Anaplasma* species have not been used for vaccination. However, it would be interesting to evaluate vaccine formulations containing other recombinant *Anaplasma* MSPs such as the *A. phagocytophilum* MSP2.

Conclusions

Anaplasma spp. MSPs interact with host cells during infection and transmission of the pathogen. Concurrent infections with different *Anaplasma* species may introduce new considerations for the diagnosis and control of anaplasmosis. Sequence similarity in MSP5 sequences among *Anaplasma* spp. may result in a reduced ability to make a differential diagnosis based on existing serological assays in infected animals and prompts the development of alternative diagnostic methods. Genetic diversity in MSPs suggests that multiple introductions of diverse strains of *A. marginale* have occurred in most geographic areas, an important consideration when designing and evaluating vaccine formulations for the control of bovine anaplasmosis.

The ideal vaccine for anaplasmosis would be the one that induces protective immunity and prevents infection and transmission of the pathogen. Current vaccines do not prevent infection and persistently infected cattle are a major reservoir of *A. marginale*, thus serving as a source of infection for mechanical and biological transmission by ticks. Although transmission-blocking antigens have not been identified from the tick vector or the pathogen, recent results suggest that antibodies to recombinant MSP1a may reduce infectivity for *D. variabilis* (de la Fuente *et al.*, 2003c), in accordance with results obtained in neutralization studies *in vitro* (Blouin *et al.*, 2002, 2003). However, further research is needed to fully understand the developmental cycle of *A. marginale* in cattle and ticks in order to design a vaccine that will prevent infection of both hosts.

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The full genome sequences of *A. marginale* and *A. phagocytophilum* are complete (Brayton *et al.*, 2005) or close to completion and should be released in early 2005 (*A. phagocytophilum* and *A. marginale* genome sequence databases are located at www.tigr.org and www.vetmed.wsu.edu/research%5Fvmp/anagenome/, respectively). This information will prove to be essential for global comparative studies of *Anaplasma* strains, which should add considerable information on the genetic diversity of *Anaplasma* MSPs. Genome sequences can be also used for *in silico* search and biological throughput screening of polypeptides with potential value for diagnosis and vaccine development.

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